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Prophylactic effect of herbal extracts on LPS-induced inflammatory response in rat hepatocytes

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Abstract

Liver is participating in the clearance of the infectious agents mainly by Kupffer cells and to a lesser extent by sinusoidal endothelial cells and hepatocytes. Lipopolysaccharide (LPS), a major component of bacteria cell wall, is an endotoxin translocated from the gut; inducing oxidative stress and acute inflammation in hepatocytes. This study aimed to highlight the prophylactic effect of naturally occurring products (*B. vulgaris* and *C. vulgaris*) against LPS-induced toxicity. RT-PCR was performed using RNA extracted from rat hepatocytes as template to determine the gene expression of COX-2, i-NOS and TNF-α. *B.* and *C. vulgaris* showed a noticeable improvement in maintaining COX-2, i-NOS and TNF- at their basal expression level; even under LPS stimulation. However, the efficacy of *C. vulgaris* was better than *B. vulgaris*. In conclusion, LPS-induced inflammatory response to rat hepatocytes could be inhibited by *B.* and *C. vulgaris* as naturally occurring prophylactics.

Keywords: *Berberis vulgaris*; *Calluna vulgaris*; Lipopolysaccharide; COX-2; i-NOS; TNF-α.

Introduction

Hepatocytes damage can be induced by biological factors (bacteria, virus, and parasites) and autoimmune diseases (immune hepatitis, primary biliary cirrhosis), as well as by the action of some toxic compounds such as lipopolysaccharide (LPS) and alcohol [1].

Lipopolysaccharide (LPS), an endotoxin, is a major glycolipid component of the outer cell wall of gram negative bacteria, made up of a polysaccharide O-chain and a biologically active lipid-A moiety, embedded within the bacterial membrane [2]. Moreover, LPS-induced cytokines mediated inflammation is widely used model for studying endotoxemia. Endotoxemia-induced toxicity is characterized by injury to various organs, including liver, kidney and the brain, and it has been implicated as a contributing factor to bacterial infection resulting in sepsis, which is one of the major causes of morbidity and mortality in intensive care units [2]. Most of the toxicities observed in LPS induced injury in the liver and systemic circulation has been attributed to inflammatory mediators produced by activated macrophages, including tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (i-NOS), and cyclooxygenase-2 (COX-2) [3-5]. Since, TNF- is the key mediator in many experimental liver injury models, an early rise of TNF-α level induces pro-inflammatory genes; including i-NOS and COX-2 [6, 7]. Persistent hepatic injury and inflammation may lead to the progressive liver damage, fibrosis, and finally cirrhosis.

Despite enormous advances in modern medicine, there are no completely effective drugs that stimulate hepatic function, offer complete protection to the organ, or aid in regenerating hepatic cells [1]. Additionally, some drugs can induce adverse or side effects. Thus, it is necessary to identify alternative pharmaceuticals for the treatment of hepatic diseases, with the aim of these agents being more effective and less toxic.

There is accumulating evidence suggesting that medicinal plants are unlimited reservoirs of drugs. Among those plants, *Berberis vulgaris* (*B. vulgaris*) and *Calluna vulgaris* (*C. vulgaris*), that are known to have an anti-inflammatory effect [8, 9]. The diversity in their active components structure makes them a useful source of novel herapeutics [8].

The current study was designed to investigate firstly, the role of LPS induced oxidative stress and acute inflammation on hepatocytes; and secondly, the prophylactic effect of herbal extracts (derived from *B. vulgaris* and *C. vulgaris*) against LPS-induced toxicity in rat hepatocytes.

Materials and Methods

Animals

Thirty four sexually mature Sprague-Dawley male rats, about 70-80 days of age (120-150g body weight), were purchased from experimental animal house, Faculty of Pharmacy, Pharos University and housed there. Animals were chosen randomly and housed in metal cages where they were grouped (six rats/cage)

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except the induction group consists of ten rats. Experimental rats were maintained at approximately 23˚C-25˚C with a 12h light/dark cycle and received laboratory basal diet and tap water for one week acclimation period and throughout the study period. All animals' experiments were preformed according to the Guide for the Care and use of Laboratory Animals, National Institutes of Health.

Animal treatment and experimental design

After the adaptation period, the total number of experimental animals was 34 male rats were randomly divided into five groups. Control group (C, n=6) was given orally normal saline; induction group (I, n=10) represents Lipopolysaccharide administrated group (a single intra-peritoneal injection of LPS in a dose of 4 mg/Kg body weight and left alive 12 hours); vehicle group (V, n=6) represents the group that was given orally 0.66 mg/kg/daily of polyethylene glycol (PEG 20%, plant solubilizing agent) for three weeks then a single intra-peritoneal injection of LPS in a dose of 4 mg/Kg body weight and left alive 12 hours; *Berberis vulgaris* group (BV, n=6) represents the prophylactic group that was given orally *Berberis vulgaris* crude extract (200 mg/kg body weight/daily) for three weeks then intra-peritoneal (IP) injection of LPS in a dose of 4 mg/Kg body weight and left alive 12 hours; and *Calluna vulgaris* group (CV, n=6) represents also the prophylactic group that was given orally *Calluna vulgaris* crude extract (200 mg/kg body weight/daily) for three weeks then intra-peritoneal (IP) injection of LPS in a dose of 4 mg/Kg body weight and left alive 12 hours. At the end of the treatment period, rats were sacrificed then blood and liver tissue were collected.

Preparation of lipopolysaccharide

Lipopolysaccharide was extracted from E. coli, cultivated in LB-medium according to what previously described, by simple non phenolic method using methanol-chloroform extraction technique [10]. The extracted LPS were compared to standard LPS purchased from Sigma by using SDS-PAGE electrophoresis. LPS bands were then visualized by silver staining to check the purity (data not shown).

Preparation of herbal crude extract

Berberis vulgaris roots and *Calluna vulgaris* shoots were collected from the fields and authenticated by Prof. Salma El-dareir, Botany Department, Faculty of Science, Alexandria University, Egypt. The dried plant roots and shoots were separated, dried at room temperature, powdered, sieved and stored for further analysis.
The dried herbs powder were exhaustively defatted with petroleum ether, then soaked with absolute ethanol (500g/1L ethanol) for three days at 37ºC with shaking at 60 rpm. The ethanolic extract was concentrated to minimum volume using rotary evaporator (Büchi, Switzerland) then lyophilized (DISHI, DS-FD-SH10, Xi'an Heb Biotechnology Co, China) to obtain a powder form. The crude extracted powder was kept at -20 C until subjected for further biochemical analysis. Prior to animal treatment the crude extracted powder was dissolved in polyethylene glycol (PEG) 4000 (20% in d. H_2O) [11].
HPLC analysis of the polyphenolic compounds in *B. vulgaris* and *C. vulgaris*:
B. vulgaris and *C. vulgaris* ethanolic extracts were analyzed using HPLC (Agilent HPLC 1100, USA) for separation and identification of their polyphenolic sub-ingredients.
Determination of lethal dose 50 (LD50) of *B. vulgaris* and *C. vulgaris* crude extract:
25 male mice, for each of *B. vulgaris* and *C. vulgaris* crude extract, were obtained from the National Cancer Institute, Cairo University, Egypt. Mice were selected with an average body weight (20-25 g). The animals were housed and had free access to pelleted food and tap water for one week for active integration. After one week, mice were divided equally into five groups each group contains 5 mice. Group (I) represents negative control or vehicle groups which were given orally polyethylene glycol (20%) and for the other four groups were orally administrated by *B. vulgaris* or *C. vulgaris* with concentration of 100, 500, 1000 and 5000 mg/Kg dissolved in PEG 20%. They were left for 72 hours. At the end of this period, there was no any mortality occurred in any group. This proved that *B. vulgaris* and *C. vulgaris* extract have no lethal effect on all mice groups at different concentrations.

Assessment of plasma biochemical parameters in rats

Plasma glucose, liver enzymes, kidney function, and total protein were measured in rats using commercial available kits according to manufacturer instructions (Spinreact, Spain).
Assessment of pro-oxidant inflammatory molecules and antioxidants in rats liver
Pro-oxidant inflammatory molecules in rats liver homogenate [such as nitric oxide (NO), thiobarbituric acid-reactive substances (TBARS), and xanthine oxidase (XO)] were assessed as previously described [12-14]. Also, antioxidants in rats liver homogenate [such as reduced glutathione (GSH), glutathione-S-transferase (GST), and superoxide dismutase (SOD)] were assessed as previously described [15-17].

Molecular detection of inflammatory markers

RNA extraction. RNeasy Mini Spin Columns (Qiagen, USA) were used for purification of RNA from cells. All extraction steps were carried out at room temperature under strictly aseptic conditions and in accordance with the manufacturer's procedure. RNA integrity was assessed by agarose gel electrophoresis. Purified RNA was converted into cDNA by RT-PCR using commercially available RT-PCR PreMix (BIORON GmbH, Germany).

Primers

The primers used for amplification of inflammatory markers (COX-2, i-NOS and TNF-α) were shown in table 1.

Table (1): Primers for amplification of COX-2, i-NOS, and TNF- cDNA.

cDNA	Primers	Fragment length (bp)	Reference
Cyclooxygenase-2 (COX-2)	Forward:5'-CTGTATCCCGCCCTGCTGGTG-3'	282	GenBank
	Reverse:5'-ACTTGCGTTGATGGTGGCTGTCTT - 3'		
Inducible Nitric Oxide Synthase (i-NOS)	Forward:5'-GACTGCACAGAATGTTCCAG-3'	308	[18]
	Reverse:5'-TGGCCAGATGTTCCTCTATT-3'		
Tumor Necrosis Factor- (TNF-)	Forward:5'- TTTCCGCGGGTTGAATGAGA- 3'	101	Gen Bank
	Reverse:5'- CTTGGTGTCCTCGCTGAGTT-3'		

PCR amplification

cDNA of each inflammatory marker (COX-2, i-NOS, and TNF-α) was amplified by conventional PCR technique using a Perkin Elmer 9600 thermo-cycler, as described previously [19]. The amplified cDNA products were run on 2% agarose containing ethidium bromide (0.5 μg/ml) by electrophoresis and visualized on a UV transilluminator for positive bands; similar volumes of amplified cDNA products of all samples were loaded in all lanes, as well as the standard DNA ladder, so that comparison of the bands was more feasible. NIH ImageJ (http//rsb.info.nih.gov/ij/index.html) was used to compare and quantify the intensity of cDNA bands for the statistical analysis.

Histopathology examination of rat hepatocytes

Specimens from rat liver were fixed in 10% neutral-buffered formalin and dehydrated through ascending grades of ethanol. After dehydration, the specimens were cleared in xylol and transferred through three changes of paraffin wax for 1-2 hours. Finally Sections (5 μm thick) were stained with Ehrlich's hematoxylin and counterstained with eosin for histological evaluation [20]. The samples were investigated by light microscope (Tech optics - Germany, with digital camera sn/t50 3882).

Statistical analysis of the data

Data obtained in the present study were evaluated statistically using SPSS version 15 (SPSS Inc., Chicago, IL, USA). Numerical data were expressed as the mean ± standard deviation and a 2-sided p-value of 0.05 was considered statistically significant.

Results

Evidence of illness in LPS-injected rats

Rats injected with 4 mg/Kg LPS showed evidence of illness by 3h, as indicated by lethargy, ruffled fur, and shivering. The posture, behavior patterns and biochemical parameters (glucose, liver enzymes, kidney function, and total proteins) were also affected, compared to control, due to LPS administration (data not shown). Approximately 10% of rats in induction group died after LPS injection.

HPLC analysis of the polyphenolic compounds in *B. vulgaris* and *C. vulgaris*:

B. vulgaris ethanolic extract had Gallic acid (3.55 mg/ml), chlorogenic acid (8.68 mg/l), 3,4-Dicaffeoylquinic acid (1.53 mg/l), 3,5-Dicaffeoylquinic acid (3.11 mg/l) and Retinol (0.315 mg/l). However, no Caffeic acid, Rutin or Tannic acid were found. *C. vulgaris* extract had lower concentration in Gallic and chlorogenic acid than that of *B. vulgaris* extract (1.264 mg/ml and 1.09666 mg/l, respectively). On the other hand, *C. vulgaris* ethanolic extract showed higher concentration in 3,4-Dicaffeoylquinic acid and 3,5-Dicaffeoylquinic acid (2.63 mg/l and 26.18220 mg/l, respectively) than *B. vulgaris* crude extract. Furthermore, *C. vulgaris* had Caffeic acid (1.20981 mg/l), Rutin (3.3469 mg/ml) and Tannic acid (1.55519 mg/ml) which were not found in *B. vulgaris* ethanolic extract.

Assessment of pro-oxidant inflammatory molecules and antioxidants in rats liver

Pro-oxidant inflammatory molecules (NO, TBARS, and XO) as well as antioxidants (GSH, GST, and SOD) in liver homogenates showed the highest concentration in LPS-induction group and vehicle group. Whereas, *B. vulgaris* and *C. vulgaris* pretreatment showed a significant decrease in concentration of pro-oxidants and antioxidants. However, the efficacy of *C. vulgaris* was better than *B. vulgaris* (table 2 and 3).

Table (2): Assessment of pro-oxidant inflammatory molecules in rats liver

Group	NO(mg/ml)	TBARS (nmol/ml)	XO (µmol/hour/gram)
Control	1.40 ± 0.03	6.06 ± 0.71	0.05 ± 0.01
Induction	2.36± 0.22 [a]	10.15±0.55 [a]	0.09± 0.01 [a]
Vehicle	2.3 ± 0.9 [a]	9.91 ± 1.28 [a]	0.085 ± 0.0 [a]
B. vulgaris	1.62 ± 0.07 [abc]	8.79 ± 0.92 [abc]	0.06 ± 0.01 [abc]
C. vulgaris	1.54± 0.03 [abcd]	8.41 ± 0.54 [abcd]	0.055± 0.01 [abcd]
P value	<0.001*	<0.001*	<0.001*

a: Significant with control group. b: Significant with induction group. c: Significant with vehicle group. d: Significant with B.V group.
*: Statistically significant at p 0.05.

Table (3): Assessment of antioxidants in rats liver

Group	GSH (mg/mg protein/g tissue)	GST (mg/mg protein/g tissue)	SOD (IU)
Control	0.01 ± 0.005	0.16 ± 0.03	0.009 ± 0.001
Induction	0.043 ± 0.018 [a]	0.45± 0.02 [a]	0.019± 0.001 [a]
Vehicle	0.037 ±0.025 [ab]	0.43 ± 0.02 [a]	0.021± 0.004 [a]
B. vulgaris	0.026± 0.0 [bc]	0.26 ± 0.02 [abc]	0.015 ± 0002 [abc]
C. vulgaris	0.022 ±0.004 [abcd]	0.18 ± 0.04 [bcd]	0.012±0.006 [abcd]
P value	<0.001*	<0.001*	<0.001*

a: Significant with control group. b: Significant with induction group. c: Significant with vehicle group. d: Significant with B.V group.
*: Statistically significant at p 0.05.

Effect of *B. vulgaris* and *C. vulgaris* on COX-2, i-NOS, and TNF-α gene expression in rat hepatocytes

Both LPS injection and the PEG vehicle administration up-regulates COX-2, i-NOS, and TNF-α gene expression in comparison to control group. Administration of *B. vulgaris* extract to pretreated BV group returned gene expression almost to control levels while administration of *C. vulgaris* extract to pretreated CV group down-regulated gene expression to levels that is lower than control expression levels (table 4 and figure 1).

Table (4): Prophylactic effect of *B. vulgaris* and *C. vulgaris* on COX-2, i-NOS, and TNF- gene expression in rat hepatocytes

Group	COX-II	i-NOS	TNF-α
Control	3.22 ± 0.3	0.22 ± 0.02	5.92 ± 0.019
Induction	6.60 ± 0.6[a]	0.50 ± 0.049[a]	20.07 ± 2.1[a]
Vehicle	5.90 ± 0.5[a]	0.74 ± 0.02[ab]	18 ± 1.2[ab]
B. vulgaris	3.08 ± 0.3[bc]	0.12 ± 0.01[abc]	6.02 ± 0.6[bc]
C. vulgaris	0.91 ± 0.09[abcd]	0.05 ± 0.004[acd]	1.09 ± 0.1[abcd]
P value	<0.001*	<0.001*	<0.001*

a: Significant with control group. b: Significant with induction group. c: Significant with vehicle group. d: Significant with B.V group.
*: Statistically significant at p 0.05.

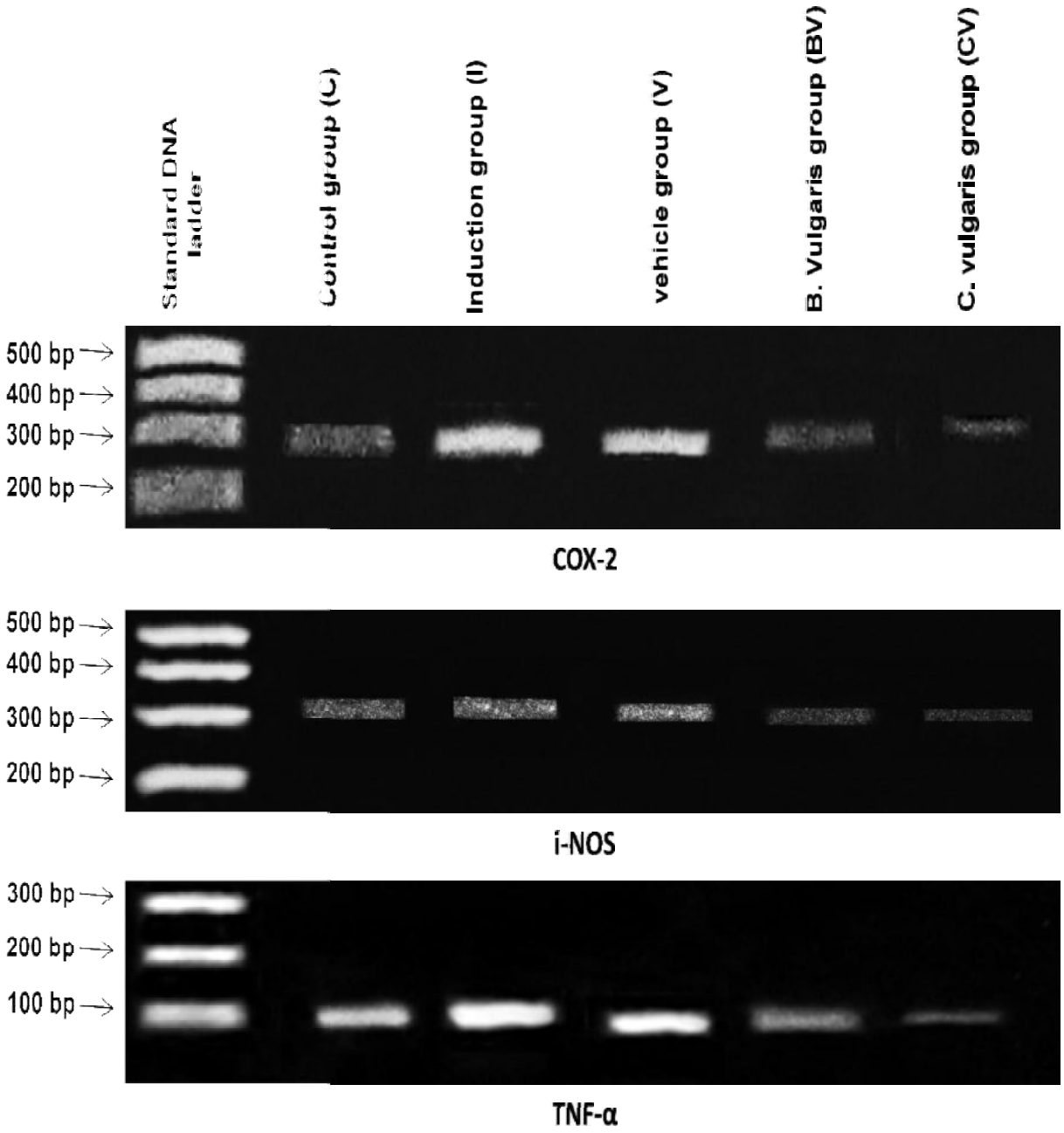

Figure 1: Bands of cellular COX-2, i-NOS and TNF-α on agarose gel of five studied groups. Bands were compared and quantified by NIH ImageJ (http//rsb.info.nih.gov/ij/index.html) in parallel with standard DNA ladder. Cellular COX-2, i-NOS and TNF-α gene expression is expressed at 282, 308 and 101 bp, respectively.

Histopathology examination of rat hepatocytes in different experimental groups

The control group had normal hepatocytes architecture showing normal portal vein, bile duct and hepatic artery. LPS-Induction group and vehicle group revealed the highest hepatocytes damage with portal inflammation and fibrosis; also hepatocytes necrosis and Kupffer cells hyperplasia were observed. Moreover LPS-Induction had acidophilic bodies which indicated hepatocytes apoptosis.

Furthermore, vehicle group revealed central vein congestion, sinusoidal dilation, and piecemeal necrosis. *B. vulgaris* pretreated group showed a decrease in hepatocytes damage than that of both LPS-induction and vehicle groups but there were portal inflammation, hepatocytes necrosis and central vein congestion, while *C.vulgaris* extract group showed the highest hepatocytes protective effect against LPS toxicity; where, there were significant decrease in portal inflammation and fibrosis associated with decrease in hepatocytes necrosis degree. Furthermore, there were decrease in sinusoidal dilatation and congestion with no acidophilic bodies (figure 2).

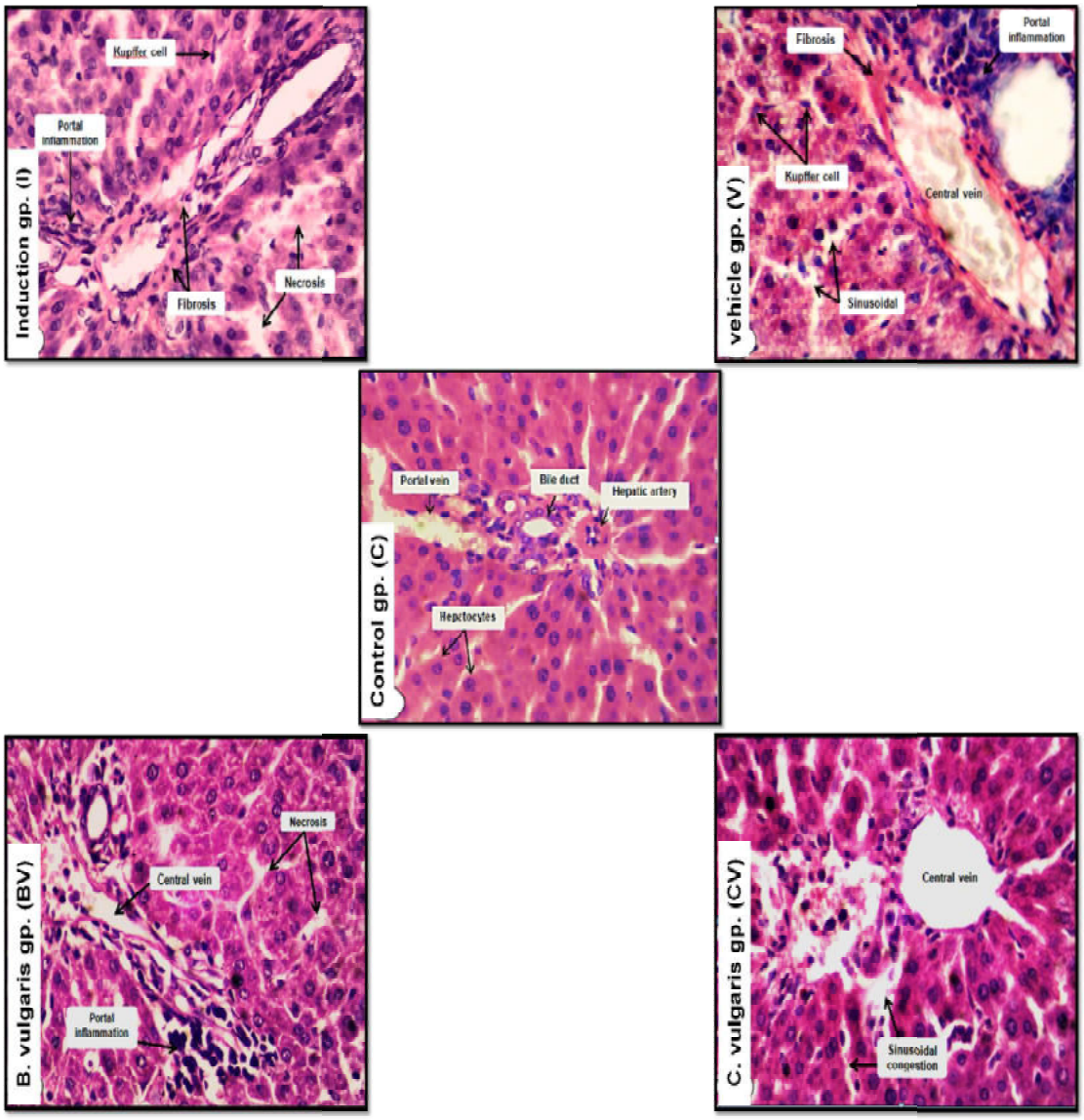

Figure 2: Histological examination of rats hepatocytes of different groups compared to control group (C). Induction group (I; LPS single i.p injection of 4 mg/Kg). Vehicle group (V; 0.66 mg/kg of 20% polyethylene glycol and LPS single i.p injection 4 mg/Kg), *Berberis vulgaris* group (BV; 200 mg/Kg orally and LPS single i.p injection 4 mg/kg). *Calluna vulgaris* group (CV; 200 mg/Kg orally and LPS single i.p injection 4 mg/Kg).

Discussion

The present study aimed to highlights two main points, firstly, the role of LPS induced oxidative stress and acute inflammation in hepatocytes; secondly, the prophylactic effect of naturally occurring

products against LPS toxicity. Lipopolysaccharide is a well-known immune-stimulatory agent that causes tissue damage and organ failure through the production of excessive and uncontrolled pro-inflammatory mediators as well as ROS generation [3, 21].

Systemic acute inflammatory reaction induces a series of responses in human and experimental animals, which generates two consequences: one is a protective response and the other induces an exaggerated systemic response termed as systemic inflammatory response syndrome (SIRS). What related to SIRS is a competitive balance of pro- and anti-inflammatory reaction [22]. Imbalance of the competition results in multiple organ dysfunction syndrome (MODS) or compensatory anti-inflammation response syndrome (CARS), both of them are harmful to organism even result in lethal systemic disorder called LPS toxicity induced septic shock [23].

Recent observations suggest that during endotoxemia, parallel with the activation of hepatic macrophages, sinusoidal endothelial cells display an increased capacity to detoxify ROS; this may counterbalance the increased pro-oxidant status of hepatic sinusoids [24]. Therefore, it is not surprising to find in our data that in LPS-induction group both pro-oxidant and detoxifying enzymes activities in hepatocytes are elevated as a defense mechanism to maintain host homeostasis.

In the present study, pretreatment of rats with *B. vulgaris* and *C. vulgaris* crude extract showed noticeable improvement against LPS induced inflammatory response, as there was a competitive struggling between liver antioxidants detoxifying mechanism and LPS toxic effect. On molecular levels *B. vulgaris* and *C. vulgaris* crude extract successfully attenuated acute phase response and hepatic dysfunction which resulted from LPS-administration after 21 days of herbal pretreatment, showing normal pro-inflammatory cytokines gene expression with moderate improvement in pro-oxidant and histological architecture when compared to control group.

The results of current research coincide with in vitro studies that showed, berberine as the most active alkaloids ingredient that effectively suppresses the expression of pro- inflammatory cytokines, including TNF-α (a master cytokine), that subsequently inhibiting downstream mediators of inflammation as i-NOS and COX-2 [25-27].

The preventive effect of *C. vulgaris* polyphenols is due to the free radicals scavenger via the induction of detoxifying enzymes and antioxidant response element [28].

Antioxidant defenses (SOD, GSH and GST) were significantly increased than that of the control due to the antioxidant effect of *B. vulgaris* and *C. vulgaris* crude extract as well as acute phase response against LPS toxicity. SOD, considered to be the first line of defense against oxy radicals, catalyzes the dismutation of superoxide radicals to H_2O_2 and molecular oxygen. Also, GSH play essential role in cellular antioxidant defenses and most importantly to remove H_2O_2 and organic peroxides.

This is owing to ROS-detoxifying enzymes that are highly elevated in response to high concentration of ROS level in induction group. However, the severity of ROS levels, which were associated with lower acute response, is highly attenuated by *C. vulgaris* rather than *B. vulgaris*. Cytotoxicity of lipid peroxidation products is reduced in part by intracellular GSTs [29]. Thus, in the present study an increase in the activities of GSH and GSTs in the liver of *C. vulgaris*-pretreated rats decreased the production of peroxidation by-products TBARS than that of the induction group; but it actually not returned to the control one and this could be due to TBARS increased significantly at 6–24h post- LPS administration to reach a maximum increase at 12 hours which was the duration in this study so it was predicted to be returned to normal one after 72 hours, similarly, as previously reported [30].

The decreased LPS-induced ROS and decreased TBARS in *C. vulgaris* group might be due to presence of caffeic acid and 3,5-Dicaffeoylquinic acid, that are known previously to inhibit both lipid peroxidation and intracellular ROS accumulation induced by LPS [31, 32].

Moreover, *B. vulgaris* and *C. vulgaris* extract reduced the generation of NO in vitro in a concentration dependent manner. Further, the high scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO, that are detrimental to the human health as excess NO is known to damage the immune system [33].

Nevertheless, It was demonstrated that pretreatment with *C. vulgaris* extract reduced lipid peroxides and nitric oxide generation and inhibited the ultra violet B (UVB) induced apoptosis and inflammation in mice [34]. That is coincided with the present study that reveals significant decrease in pro-oxidants biomarkers (TBARS, NO and XO) levels than that of the induction group.

B. vulgaris and *C. vulgaris* successfully down regulated i-NOS gene expression; thus nitric oxide should be returned to the control level. But it was noticed that it was higher than that of the control, although it is lower than the induction group. The suspected interpretation is that there was an increase in Xanthine oxidase enzymes activity due to LPS effect than that of the control. It is known that, Xanthine oxidase is involved in the generation and metabolic fate of nitric oxide (NO), an important component and regulator of the immune response towards LPS endotoxin or infection [35]. Both, *B. vulgaris* and *C. vulgaris* decreased XO activity level than in induction group.

Histological study of liver from *B. vulgaris* and *C. vulgaris* groups revealed a decrease in hepatocytes necrosis and inflammation than that of LPS induction group. But *B. vulgaris* crude extract failed to prevent central vein congestion and sinusoidal dilation when compared to *C. vulgaris*. That could be due to presence of caffeic acid, rutin and tannic acid content in *C. vulgaris*.

Therefore, *C. vulgaris* preserved the structural integrity of hepatocellular membrane, with no signs of hemorrhage or apoptosis. However, a slight sinusoidal congestion and dilatation are noticed.

In conclusion, our obtained data showed clearly that, LPS functions as a pro-inflammatory and pro-oxidant mediator inducing apoptotic cell damage that results in hepatocytes dysfunction. That has been

confirmed by morphological, molecular and biochemical changes. The prophylactic effect of *C. vulgaris* against LPS toxicity in hepatocytes was higher than the effect of *B. vulgaris*. Therefore, *C. vulgaris* could be considered as a potent preventive agent against hepatotoxicity.

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